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Bacteria isolated from maples.

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BACTERIA ISOLATED FROM MAPLES

By

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INTRODUCTION

In response to recent, wide-spread, public concern over the decline of sugar maple trees in Massachusetts, a research project (McIntire-Stennis Project 1) was undertaken in 1963 at the University of Massachusetts. The investigation was divided into Subprojects, each devoted to a particular aspect of the possible causes of poor health of maple trees. These facets included: insect injuries, nutritional factors, toxicity of metal ions in the soil, toxicity of road salts, macro-ecological factors in the site, soil structure and drainage, environmental injuries including drought, as well as pathological conditions and agents such as viruses, nematodes, fungi and bacteria.

Bacteria have been found in association with decay in living trees (Shigo, 1965). It is unknown whether these bacteria are always saprophytes, or whether they contribute to the declination of the trees they inhabit. Perhaps bacteria that are normally saprophytes can become aggressive when they inhabit trees that are under environmental stress, such as that imposed by water shortage.

The wetwood disease has been studied in elms and poplars by Carter (1945) and Seliskar (1950), respectively. In each case the pathogen was shown to be a bacterium, but a separate genus and species, and in each case a bacterium that had not previously been described. It has been assumed that a bacterium causes the wetwood disease in maples. Except for the presence of slime flux, which does not always occur with the disease, the symptoms of wetwood are similar to those of "decline".

The present report derives from inquiry during 1965-67 into the presence and possible role of bacteria in maple trees.

SECTION ONE

BACTERIAL ISOLATIONS: DISCOLORATION AND DECAY

Review of the Literature

Discoloration of wood often indicates decay; however there are other reasons for its occurrence. Boyce (1962, pg. 494) reports that superficial chemical stains often occur on sawed lumber; the sapwood of birches, black cherry, and maple stains reddish-yellow or rusty color. Also a greenish-black stain either paralleling the grain as streaks or as a solid, mass discoloration may develop in living sapwood of sugar maple and other hardwoods. This type of stain is called mineral streak. Lorenz (1944) and Roth (1950) concluded after many isolations from discolored wood that, while discoloration is almost always associated with decay, it is not necessarily so; some discoloration is of a chemical, non-biotic, origin. Shigo (1965) in his studies of decay and discoloration in Northern hardwoods finds no discoloration that is not at least closely adjacent to that which is of biotic cause. On the other hand, he has isolated bacteria from non-discolored tissue, but that too, is adjacent to discoloration from decay organisms.

Boyce (1962, pg. 345) discussed discoloration as one of the early signs of decay. In the decay process the initial change in color that occurs while the wood is still sound is referred to as the "incipient stage" of decay. The discoloration may be slight to marked. Good et al (1955) found stain from the fungus Polyporus glomeratus Peck to extend several feet in advance of the

mycelium. They postulated that the stain was a reaction to toxins released by the fungus that diffused through the wood.

Since decay often occurs in the heartwood of the tree, it is important not to confuse the color changes associated with decay with those natural to the heartwood. Loosely used, the term "heartwood" describes nothing more then the darker, central wood of the tree. However, a more technical description would include the specification that there are no living cells in the heartwood. In some trees, such as oaks, there is a sharp demarcation between light, living sapwood and the darker heartwood. Other trees, such as sugar maples, do not have this sharp line. Good et al (1955) studied the heartwood of maples and found that a slight, gradual change in coloration from the sapwood to the interior wood was correlated with a gradual decrease in the number of living cells. They reported finding living cells in wood as old as 115 years. However, no one generalization is true. A wide range of coloration is natural to the heartwood, even of trees within a single species.

Bacteria have been studied only infrequently in the decay and discoloration process. When they have been studied, they usually have been lumped under the category "bacteria" with no further differentiation attempted.

Schmitz (1919) inoculated the wood of two broadleaved trees and two conifers with bacteria and fungi isolated from the discolored interiors of decaying trees of the respective species. He observed that bacteria accelerated the rot process considerably, and more markedly so in the case of the white rots than of the brown. He concluded that between the bacteria and the fungi there was some interaction which was not understood and which needed further study.

Eades and Alexander (1934), in their work on Western red cedar, found an abundance of moulds, bacteria and yeasts in what they called "normal dark heartwood," which, however, was not decayed.

Lorenz (1944) studied the association of microflora with discoloration resulting from increment borings. He found all increment borings to have resulted in stain in the tree. Some of the holes had been disinfected immediately after boring and plugged with dowels. Fungi and bacteria were isolated from most of the discolorations regardless of the treatment after boring.

Roth (1950) studied discoloration in yellow-poplar. Inoculations were made with bacteria and fungi isolated from discolorations of veneer logs into bore holes and axe cuts of large, healthy trees. His bacterial isolates were of a few basic types, but none was isolated consistently from any one type of discoloration. Controls consisted of holes sterilized with 70% ethanol. All the treatments resulted in about the same range of discoloration; because of this he concluded that the discoloration was not of a bacterial or fungal origin, but the result of something else, possibly a chemical oxidation.

Basham and Taylor (1965) report isolating fungi and bacteria from 38% of the samples taken from discolored heartwood of sugar maples. However, in this report only 53% recovery of microorganisms was found in decayed heartwood.

Shigo (1965) studied the interaction of bacteria and fungi in beech, birch and maple. He studied these organisms as they succeeded in common infection courts: branch stubs, mechanical wounds, and squirrel wounds. He found that once wood tissues were exposed, a succession of events began, one of the first

of which was invasion by bacteria and non-hymenomycetous fungi. These were associated frequently with discoloration. The hymenomycetes, the decay fungi, invaded only discolored tissue. Exceptions to this rule were found only with the hymenomycetes Poria obliqua (Pers.) Bres. and Polyporus glomeratus Peck which were found as primary invaders along with bacteria and non-hymenomycetes. McCreary et al (1965) identified some of the bacteria isolated in Shigo's (1965) study as belonging to the genera Bacillus, Pseudomonas, Xanthomonas, and Erwinia.

Materials and Methods

Bacteria were isolated from the tree by one of two techniques, depending upon whether the sample had come from a branch or from a trunk.

The branch samples were always less than 3/4 inch in diameter and were cut into sections about four inches long when removed from the tree. One end of the sample was dipped into 70% ethyl alcohol in a pint jar, removed, held horizontally and then ignited. The alcohol burned off in about ten seconds. A knife was heated in a Bunsen burner flame, cooled in a separate jar of alcohol and then likewise ignited to burn the alcohol off. With this knife the bark was peeled back about 1 1/2 inches from one end of the twig. As far as possible, the peeling was done in such a way that only the very end of the freshly exposed wood was touched by the knife blade. With another knife, cleansed in the same way, a "V" cut was made in the exposed wood perpendicular to the length of the twig and a number of cuts parallel to one side of the "V" were made about 1/16 inch apart. Tweezers were then cleansed in the same way as the knives. The individual chips of wood were lifted with the tweezers from the twig sample and put into petri dishes.

Each petri dish contained 15 ml. of potato dextrose agar or peptone, yeast-extract, dextrose agar prepared as described below. There were usually six chips of wood per dish.

The trunk samples or samples from large limbs were slabs cut from the tree with a mallet and hatchet, and were not smaller then three inches square and one inch thick. With these slab samples a previously unexposed surface for culturing was obtained in a different way. A hatchet and mallet were used to split the sample in two perpendicular planes. The surfaces below where the hatchet was wedged in were untouched. From the corner created by these surfaces a "V" would be cut, with parallel cuts to form individual chips in the same way as on the branch samples.

The cultures were incubated at 28°C. and visible bacterial growth usually occurred around the chips within 24 hours, if any occurred at all. By Gram stains an estimate of the different kinds of bacteria growing around any one sample was made and two successive streak plates were made in order to obtain each type of bacterium in pure culture.

The ability of the bacteria isolated to grow on wood without any other organisms present was tested in two ways. In one method disks, 1/4 inch thick and 1 inch in diameter, were cut from branches of the same diameter and steam sterilized at 15 pounds for 15 minutes. They were placed in sterile petri dishes with a few ml. of sterile distilled water and the bacteria under study were spread across the surfaces of the disks. In the other method chips were cut from un-decayed branches in the same way as described above for the isolation of bacteria. These chips were placed on nutrient agar and incubated for 1 week at 28°C.

Chips showing no bacterial or fungal growth at the end of this time were believed to be "sterile", that is to have no other living matter than the wood itself. The chips were then placed on water agar and the bacteria under study were spread across the surfaces of the chips. In both of these methods incubation, after the introduction of the bacteria under study, was at 28°C.

The Gram stains and the size measurements taken from them were made from cultures grown on trypticase soy broth and incubated at 28°C for 48 hours. The characteristics of growth in a liquid were made from the same cultures. Morphology of the colonies on solid agar was studied from cultures growth on trypticase soy agar for 48 hours. Utilization of each of the carbohydrates tested was determined in a 1% solution in a yeast-extract,peptone broth with brom cresol as an indicator. Small, inverted tubes in the broth were used to trap gas, if any were produced.

Further methods for studying the bacteria followed the recommendations of the Society of American Bacteriologists in Manual of Microbiological Methods.

Results

Cultures of 3 bacteria isolated from separate afflicted limbs of a sugar maple which was suffering from severe dieback. Half of the crown was leafless and the other half had sparse foliage. The tree was large (about 3 feet d.b.h.) and was located on the tree belt adjacent to the grounds of Deerfield Academy in Deerfield, Massachusetts.

The cultures were made from the interfaces of the healthy wood and the decaying wood in the dying limbs. Since the limbs were quite large in this case, sometimes the samples were slabs. The decay interface was identifiable by the drier, coarser texture of the decayed wood compared to the moist, creamy appearance of the healthy wood. Sometimes in the area of the interface there were blackish or greenish lines which ran either transverse to the long axis of the branch or, in some cases, parallel to it for at least a few feet. In the latter case one side of a given branch might be alive while the other side was decaying. In either case the longitudinal separation of the branch portions that were wholly decayed from the portions containing only healthy wood was only a few feet.

Other isolates were taken from the flare of the trunk in the tree described above. Green streaks were observed in and around various degrees of decay in the sapwood. Verticillium sp. was found in the original isolates along with the bacteria.

The six different bacterial cultures that were studied proved all to be motile, small, Gram-negative rods. They released a water soluble green pigment into the culture media. They are believed to be species of the genus Pseudomonas.

A number of cultures were made from cankers of a sugar maple on Second Street in Turners Falls, Massachusetts. The cankers were small about 1/2 to 3/4 inch in diameter, and had been bleeding sap during the late spring. The necrotic tissue of the cankers was confined to the present year's wood, and the bark covering the cankers was still intact. The sap appeared to have oozed through the bark without breaking it. The advancing edges of the cankers were green. The tree was suffering dieback in the crown; however this may well

have been caused by poor site. The tree belt was narrow and the soil compacted.

Two isolates showed up consistantly in samples taken from the cankers. They differed in their appearance on nutrient agar, one colored yellow and translucent, the other creamish. They also differed in their use of lactose, not using it at all, and producing a slight acid reaction respectively.

Both cultures were small, motile, Gram-negative rods, ranging between 0.4-0.7u x 1.2-2.0u, mostly 0.55u x 1.6u. Both cultures produced acid and no gas from:

l(+)arabinose

cellobiose

d-glucose

d(+)galactose

fructose

d-mannitol

d(+)mannose

sucrose

d(+)xylose

Gelatin was weakly liquified. Starch was not hydrolyzed. Both cultures grew well on a mineral-salts medium with only ammoniacal nitrogen. Nitrite was produced from nitrate with the formation of no gas.

A series of isolates was made from the branches of a sugar maple in West Springfield, Massachusetts. This tree was also suffering from dieback. It was located on Ashley Street in a tree belt with healthy trees on each side.

The tree had been dying back for several years (Swift, personal communication). Cultures had been previously taken from the tree in search of a vascular pathogen, and cultures were taken again in this study for that reason, yet no vascular pathogen was found. The bacterial isolates were obtained from dying branches, again in the vicinity of the advancing margin of decay.

Three separate visits to this tree and preliminary studies yielded 30 cultures. These were reduced finally to two basic types.

The first type was a large, motile, Gram-positive rod with spores. The spores were about the same size as the vegetative cells. When subjected to the Gram-stain procedure they became dark at either end and remained clear in the middle. They produced only very slight acid from dextrose by the end of 72 hours. These characteristics indicate the genus Bacillus.

The second type was a small, motile, Gram-negative rod. Its size ranged from 0.5u - 0.8u x 2.2u - 3.5u, being approximately evenly distributed within these limits. The colonies on agar were creamish-white. Acid was produced from a wide range of sugars (those listed above for Second Street isolates) except lactose. No gas was produced. Gelatin was hydrolyzed but starch was not. Nitrite was produced from nitrate without the formation of gas.

Cultures were isolated from five separate branch stubs on healthy maples growing at different locations on the University of Massachusetts campus in Amherst. Healthy branches, all about 1/2 inch in diameter, were pruned from the trees to be sampled and the resulting stubs were then left without treatment to permit microorganisms to enter naturally. Two weeks after the pruning the branch stubs were removed from the trees and taken to the laboratory

to isolate into culture any invading microorganisms. While the wood on the immediate distal ends of the twigs had a dry, loose-grained appearance, there was no sharp differentiation between healthy and decaying wood. The wood on the immediate distal ends of the twigs, about the last 1/8 inch, yielded prolific growth of decay fungi. The next 1/8 inch down the branch gave both fungi and bacteria upon culturing. The samples were grown on both potato-dextrose agar and yeast-extract-peptone-dextrose agar.

The bacteria cultured from the branch stubs were all motile, Gram-negative rods, 0.36 x 1.32u. The colonies had a creamish appearance on agar. Many different sugars were fermented with the formation of acid but no gas. Lactose was utilized with the formation of only slight acid. Growth on glucose salts medium was weak. Gelatin was hydrolyzed but starch was not. Nitrite was produced from nitrate without the formation of gas.

All of the cultures isolated (with the addition of Sarcina sp., Bacillus sp., and Aerobacter sp., isolated respectively from human skin, soil, and sewage) grew readily on sterilized wood blocks and also on wood chips which were prepared as described above under Materials and Methods.

None of the organisms tested were found to be catalase negative, to use cellulose as a carbohydrate or to produce pectinases.

Discussion

Technique was found to be of great importance in the isolation process. Hartley et al (1951) discussed research on the occurrence of bacteria in normal, non-discolored sapwood of trees; considering the research done by Carter (1945), Seliskar (1950), Clausen (1949 and 1952) and Roth (1950), he states, "The contradicitions in both negative and positive findings call for some careful checking of isolation technique before final conclusions can be drawn."

While the use of one knife and one supply of alcohol is almost always successful in the isolation of fungi from contaminated wood, it is not so with bacteria. Apparently, small pieces of bark that stick to the knife blade release bacterial spores into the alcohol supply. These spores contaminate the instruments subsequently dipped into this alcohol, even though they then are flamed. This does not happen if two knives are used, with a separate supply of alcohol for each knife. Isolations from non-discolored wood were always made as a control when isolations from discolored wood were attempted.

The bacterial species found in the decaying branch stubs of otherwise healthy trees might be considered saprophytic, though the possibility that these bacteria are parasites in the initial stages of infection cannot be ruled out entirely. When the bacteria of these branch stubs are compared to those isolated from unhealthy trees, it gives an indication of which bacteria from the unhealthy trees contributed to that condition, and which were there merely because the tissues were dead. Thus, the bacteria isolated from the Ashley Street tree and one, possible both, of the isolates from the Second Street tree bear close resemblance to the bacteria found in the branch stubs of the healthy trees around campus.

A positive identification of the genus of these bacteria would require more data; in particular, it would require a knowledge of the distribution of the flagella, something which was not determined conclusively in this study. However, the data point to the genus Pseudomonas. The genus Erwinia as described by Skerman (1959, pg. 93) also could fit the data presented except that this genus commonly uses lactose, producing acid, while Pseudomonas does not (ibid, pg. 61). The weakly acid reaction with lactose by the cultures from the branch stubs on the healthy trees could have been an artifact arising from the breakdown of substances other then lactose in the culture medium. If the use of lactose be considered definitive for the differention of these genera, then, based upon the data presented, Pseudomonas was present in every sample studied. Also, then, this would mean that the one isolate that did use lactose, from the canker on the Second Street tree, may have been Erwinia.

Xanthomonas, in the family Pseudomonadaceace, is a genus commonly found on plant material, and was reported by McCreary (1965) in decaying trees. This genus commonly uses lactose (ibid, pg. 62). However the yellow, non-watersoluble pigment characteristic of this genus was not observed in any of the cultures.

Members of the genus Bacillus, observed in the advancing margins of decay in the Ashley Street tree are common saprophytes on organic matter (Breed et al 1957). McCreary (1965) also found this genus on the margins of decay.

The role of bacteria in the decay process is not known. The fact that they can act as saprophytes, often causing little harm to healthy trees they inhabit,

does not preclude the possibility that on weakened trees they may become aggressive, and contribute to the decline of the tree. Such phenomena are well known for certain fungi. Hartley et al (1961) speculated that bacteria, which generally prefer a higher pH than fungi, grow on the undecayed wood when it is first open to contamination, lowering the pH with their acid waste products until it reaches an acidity favorable for the rapid growth of the fungi. Shigo (personal communication) has pointed to the fact that thiamine, an important growth requirement of many Hymenomycetes, is produced by Bacillus spp. and Pseudomonas spp. in pure culture. This may explain why bacteria precede fungi in the decay process.

SECTION TWO

WETWOOD

Review of the Literature

The wood inside certain living trees appears wet under particular conditions. In the incipient stages of certain white rots, water-soaked areas occur in the heartwood of freshly felled trees (Boyce, 1962, pg. 348). This condition can also occur independently of fungal action (ibid). These conditions are often descriptively called "wetwood." However, with the description of the wetwood disease of elms caused by bacteria (Carter, 1945) and reports of similar bacterial diseases in a large number of other trees, (Crandall et al, 1937; Hartley and Davidson, 1950; Seliskar, 1952), it would avoid confusion to reserve the word "wetwood" for just the diseases incited by bacteria.

The most easily observed symptom of the wetwood disease is slime flux on the side of the tree. Some workers have concluded that slime flux is a physiological response of the tree, not to a pathogen, but to some artificial environmental condition (Ogilvie, 1924; Guba, 1934; Guba 1942). Other authors refer to slime flux as occurring only where there is wetwood. Dodge (1937) says, "A tree should not be diagnosed as suffering from slime flux unless there is a 'wet wood' condition of the heartwood and unless the bleeding of moisture from the tree issues from this heartwood."

Crandall et al (1937), Carter (1945), and Seliskar (1952) frequently noted the presence of slime flux on wetwood-diseased trees. They found fluxing to occur often through such openings in the tree as pruning wounds or frost cracks. Fluxing from branch crotches is common. The exudate dribbles down the side of the tree where it is open to the action of numerous secondary organisms, such as bacteria and yeasts. Often a white deposit is left on the bark as the flux dries.

In wetwood of elms, the interior, water-soaked wood of the diseased tree is characterized by a dark brown discoloration which usually is confined to the inner sapwood and heartwood, but may appear in the current season's wood as bands or streaks. Bacteria fermenting the sap in the trunk produce large amounts of gas which, being confined, develops high pressures. Sap accumulates in abnormally high amounts in the affected wood and, because of the high pressures, sap is often forced out through trunk openings. (Carter, 1945)

In elms the foliage may wilt. This is believed to occur because of toxic substances in the fermented sap. More frequently, though, yellowing and browning of the foliage occurs, followed by leaf abscission and branch dieback. General decline may occur in the larger trees. (Carter, 1945)

The symptoms of wetwood in maples as described by May (1961) are similar to those of elms. May adds, though, that not all trees show slime flux, "Many...grow poorly and show symptoms of decline, such as small leaves, dying leaf tissue, and dieback of shoot tips." He also speculates that the cause of the disease is a bacterium. Hartley and Davidson (1950) state that the disease is only infrequently encountered among maples.

The precise role of bacteria in this disease has been the subject for speculation (Hartley et al, 1961). One hypothesis is that the cells of the parenchyma die a natural death and that saprophytic bacteria cause the changes which make the wood appear wet. A second hypothesis is that the death of the parenchyma is hastened by weakly parasitic bacteria, which may or may not be present as saprophytes in the tree, but in any case are unable to attack living cells until the latter become senescent. The fact that wetwood sometimes occurs in new sapwood would tend to support the second hypothesis. However, mixtures of different bacteria have been found in wetwood. Therefore, it is possible that some of the wetwood characteristics are due to an action of, or an interaction with the secondary organisms. Seliskar's (1950) isolates from wetwood of poplars did not produce the gas in culture which is so obvious a symptom in nature. It is possible that the gas is produced by secondary organisms, independently of the wetwood, or that gas can be produced only by certain combinations of the organisms and not by any one alone.

The only intensive studies into the etiology of wetwood, those by Carter (1945) and Seliskar (1950), revealed as primary pathogens species of bacteria never before described. In each case, however, inoculations of the pathogens into nursery and greenhouse trees failed to produce the full expression of the disease on any single tree.

Materials and Methods

Samples were taken in two ways to test for pathogens in trees affected with wetwood. The first way was the slab technique which was described in Section I. This was used to take samples from the trunk. The second way was the use of an increment borer to obtain a boring from the trunk.

The increment borer was used in a way that was designed to introduce as few contaminants as possible. Two techniques of decontaminating the borer were used. In one technique, the borer was decontaminated for repeated trials in the field by dipping and flaming in 70% ethyl alcohol. In the other technique the instrument was sterilized in the laboratory with steam at 15 pounds pressure for 15 minutes. It was then placed in a sealed, sterile canister until it was needed. Of course only one sample could be taken on each trip from the laboratory with this latter technique. With both of these techniques the boring was preceded by the removal of the outer portion of the bark, and a swabbing with 70% alcohol on the exposed tissue in the area where the boring was to be made. As soon as the boring had been removed from the tree it was placed in a sterile, screw-top test tube and taken back to the laboratory for study.

To culture the microorganisms from the slabs, chips were cut and incubated as described in Section I. Two techniques were used to culture microorganisms from the borings. One was to place short sections of the boring on nutrient agar, and incubate, observing what grew around the sections, in the same way as was done with chips cut from branches or slabs. The other way was to put the sections from the boring into a test tube with 10 ml of sterile water and let them stand for 20 minutes. Then a series of 1 to 10 dilutions was made from the original water and boring, and 0.1 ml of each of these dilutions was plated on nutrient agar. Further studies were carried on from the plates of the dilution that showed good separation of colonies. Streak plates were made from each of the isolates to assure that each was a pure culture.

The methods for the further study of the bacteria described in Section I were used also for studying the isolates in this section.

Results

The bacterial flora occurring on the insides of two trees affected with wetwood was studied. One tree was a Norway maple which had no slime flux and the other was a sugar maple which showed flux.

The Norway maple with wetwood was located on the tree belt along Route #9 in Hadley. At the time of observation and sampling, August 1965, it showed severe leaf scorch, all the leaves on the tree were affected, and there was some branch dieback. The crown had been pruned flat across the top and the main trunk had been forked to prevent interference with electrical wires which passed through and over the tree. Its diameter at breast height was 18 inches. The trunk of the tree was about 2 feet from the edge of the highway and 3 feet from the highway's edge the level of the ground dropped 18 inches.

In addition to the pruning wounds the tree had a large crack extending 6 feet up from the ground along the trunk. At the time of sampling this crack was almost entirely healed over.

Cutting into the healed crack revealed a thin strip of discolored wood approximately 1/4 inch wide until the cut was 3/4 inch deep where the discolored wood expanded rapidly, following the annual ring at that depth around the tree. This discolored wood was a deep reddish-brown. A water-like fluid issued about 25 ml during the first 5 minutes and another 25 ml in the succeeding half hour.

Samples were taken as slabs cut from the interior, discolored wood of the trunk and also as an increment boring, through use of the instrument previously sterilized by steam.

Two types of Gram-negative rods showed consistently in the isolations. One produced yellow colonies on nutrient agar while the other produced cream-colored colonies. Further study revealed the following:

Small, motile, Gram-negative rods, occurring singly, mostly 0.3u x 1.41u ranging from 0.2u - 0.5u x 1.1u - 2.0u were found. Growth in nutrient broth was turbid and abundant and there was no significant surface growth or sediment. Colonies on nutrient agar were circular in form, raised in elevation and had entire margins. They were an opaque, creamish color. Gelatin was not liquified, starch was hydrolyzed and there was good growth on glucose-salts medium. Nitrate was reduced to nitrite with the formation of gas. Acid and gas were produced from l(+)arabinose, cellobiose, glucose, d(+)galactose, fructose, lactose, d-mannitol, $d(_+)$mannose and sucrose. Neither acid nor gas was produced from $d(_+)$xylose, $d(_+)$raffinose or i-inositol.

The other isolate was also a small, motile, Gram-negative rod, occurring singly. It was approximately the same size as the isolate described immediately above. Growth in nutrient broth was turbid and abundant with no significant surface growth and a flocculent sediment. Colonies on nutrient agar were yellow, circular in form, raised in elevation and had entire margins. Gelatin was not liquified and starch was not hydrolyzed but there was good growth on glucose salts medium. Nitrate was not reduced to nitrite. Acid but no gas was produced from all the carbohydrates tested in the above isolate except i-inositol, which was not tested.

The second tree that was affected with wetwood was on Warner Street in Turners Falls. This tree had been pruned to permit electrical wires to pass through it, and the center trunk had been cut off at 10 feet above the ground for this purpose. The diameter of the trunk at breast height was 15 inches, while at 10 feet, where it had been pruned, it was 9 inches. Two secondary branches had been permitted to develop and replace the central trunk, and both of these were approximately 8 inches in diameter. Extensive fluxing occurred from where the trunk had been cut off and there was a large area of whitish deposit on the bark below this crotch. When the tree was examined in June 1966 there was a moist area of bark where the tree was still fluxing within the area of whitish deposit. The pruning throughout the crown had removed any branches that had died back, if there had been any. The foliage showed no scorch. Samples were taken from this tree with an increment borer sterilized with alcohol.

The greatest number of isolates from this tree were spore producers, large, motile, Gram-positive rods. Spores were spherical to cylindrical, central to terminal, varying with the culture. Infrequently there showed up among the isolates small, motile, Gram-negative rods. These produced a water-soluble, green pigment in culture.

The bacteria studied in this section had the same ability to grow on wood chips as those described in Section I. They were also found not to produce pectinases or use cellulose as a carbohydrate.

Inoculations, as described in Section I, were made into trees with the bacteria isolated as described in this section.

Discussion

The Norway maple which was found to have wetwood was discovered only by accident, for the initial cuts that resulted in the release of liquid under pressure were made to study decay and discoloration in this tree. Wetwood was not expected here. Indeed, wetwood without slime flux is hard to detect, since the symptoms closely resemble those of the general decline which is so prevalent in this area.

The Norway maple in Hadley was not unique in its environment in being affected with leaf scorch and dieback. Other trees along the highway in this area were affected similarly. Considering the drought and the marginal environment of these trees, their poor health is not surprising. From the decline of the tree that did have wetwood the conclusion must be made that, at most, the wetwood contributed to the decline. Although in future years, with the return of normal rainfall, the trees in the surrounding environment may return to a normal, healthy state, the one affected with wetwood presumably would remain diseased.

The sugar maple investigated in Turners Falls also showed no symptoms that could be attributed exclusively to the presence of bacteria in the trunk, other then slime flux (which is not very harmful in itself). However, dieback could have been present before the tree was pruned. Also, any foliar symptoms would have been more evident toward the end of the summer. This tree was not surrounded by other declining trees.

The bacteria isolated from this tree bear close resemblance to those commonly encountered in the advancing margins of decay, those that are saprophytes. The surface, where the trunk had been cut off 10 feet from the ground, offers an ideal infection court for decay fungi and other microorganisms that might enter a tree.

Perhaps the bacterial action that goes on in the advancing margins of decay is not much different from that of wetwood, the difference lying in the degree of infection or in the amount of oxygen available.

The isolates from the Norway maple in Hadley are of a different nature. They are not so easily compared to the saprophytes. The genus Erwinia as defined in Breed (1957) would apply to these isolates if they were able to "Invade the tissues of living plants and produce dry necroses, galls, wilts, (or) soft rots." The determination of this depends on assessment of nursery inoculations. Other than this Breed (1957) describes Erwinia as motile rods which normally do not require organic nitrogen for growth, attack various carbohydrates with the production of acid and gas, hydrolyze gelatin and starch variably and sometimes produce nitrites from nitrates.

In the key to the genus Erwinia there are two species that do not produce soft rots or liquify gelatin. One has "luxuriant growth"; the other, not. The species with luxuriant growth is E. salicis Day, a bacterium that produces a disease in the wood of willows in England. E. salicis does not hydrolyze starch and does produce acid from a range of sugars, but not (and here the similarity with any of the isolates described above ends) from arabinose or fructose. However the species of Erwinia that produces wetwood in elms is keyed under another primary category, "Pathogens that normally cause soft rots...," so further identification must depend on further study.

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APPENDIX

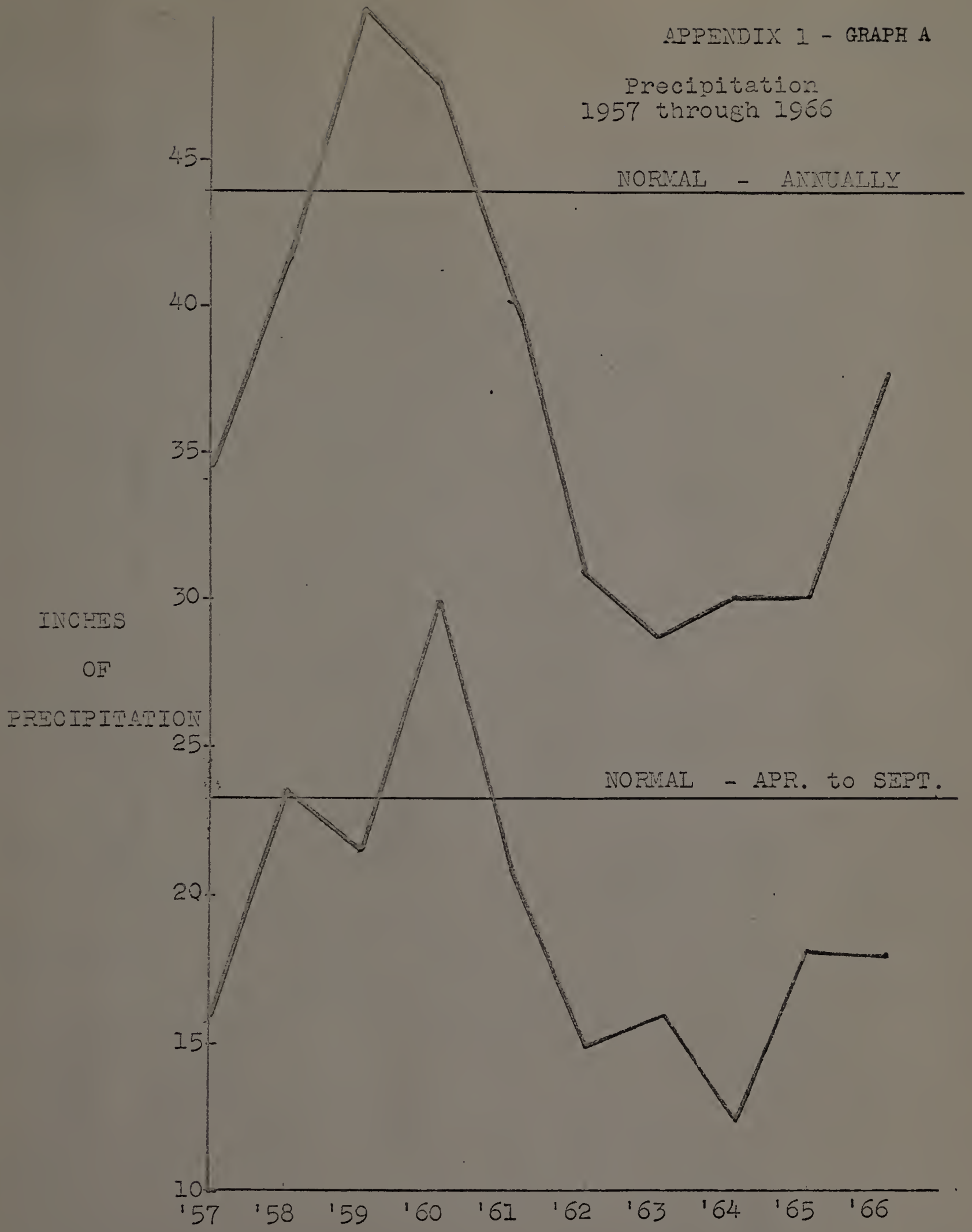
ATTEMPT TO WEAKEN NURSERY MAPLES FOR INOCULATION WITH BACTERIA

Review of the Literature

Since at least 1962 the Northeast has been in the grip of a drought, although recent indications show it to have been modified during 1966 (N.Y. Times, 1966a; N.Y. Times, 1966b; Newsweek, 1966; U.S. News and World Report, 1966). The records of the Amherst weather station through the summer of 1966, as summarized in Graph A, show rainfall to have been far below normal (Barton, 1954-57; Kleiss, 1957-61; Johnson, 1961-66). Since then rainfall is again approaching normal. The precipitation from October 1966 through February 1967 was only 2.42 inches below normal (Gricius, 1966-67).

Trees respond to drought by decreased growth. Sinclair (1964-65) compares the growth data presented by Hibben (1962), Ross (1964), and Staley (1962) for three northern hardwoods and finds a significant correlation between radial increase and growing season rainfall. Westing (1966) interprets these data as showing a 1-year lag. Friesner (1949-50) and Miller (1951-52) found that rainfall during the growing season had a direct influence on radial growth, and this influence was more marked for rainfall in the early part of the growing season. Kramer (1962) reports that summer wood formation is dependent on the supply of water, at least for loblolly pines. Large cells characteristic of spring wood are formed as long as water is readily available.

APPENDIX 1 - GRAPH A



DATA: Barton, 1954-57
Kleiss, 1957-61
Johnson, 1961-66
Gricius, 1966-67

Thus, an earlier, drier summer would result in earlier production of summer wood and less spring wood. Late summer rains cannot reverse this process. Gluck and Agerter (1962) follow radial growth as a function of rain and conclude that numerous small rains during the growing season are more beneficial to growth then the same total of rain in a few large rains. This is strikingly true, they say, for trees with a shallow root system. Thus, "drought" conditions may be brought on a tree by a redistribution of rainfall within the year, or even within the growing season, while the annual precipitation total does not change. In any case, Sinclair (1964-65) concludes that a dramatic reduction in the annual radial increase precedes the more obvious foliar symptoms that develop on the tree in response to drought.

Scorch and early abscission are often evident the same year as a drought, but branch and twig dieback do not become alarming until about two years after a year of low precipitation (Westing, 1966). Unpublished records of the Shade Tree Laboratories show that in the analysis of two thousand unhealthy maples over the 1955-1964 period there were two peak years in the number of diagnoses. In 1959 and in 1964 the number of maples diagnosed was 66% and 65%, respectively, above the ten year average. In both of these years the increase in diagnoses due to living pathogens was only slight and insignificant. There were, however, large increases in diagnoses within the categories "civilization" and "unknown," large enough to account for the total increases. One could say that the maples were more sensitive to the abuses of their environment during these two years, an observation which

may be tied significantly to the fact that each of these years comes two years after a drought year.

Drought damage is often connected with ecological factors that influence the tree unfavorably. Banfield (1955) points out some of these situations which have led to the decline of maples. Thus, when assessing the influence of drought on a tree, we must consider factors in the environment which have the ultimate result of limiting the water supply to the leaves: type of soil and its water holding capacity, exposure of the tree to direct sunlight and drying winds, and physical injuries to the roots. The possible influence of biotic factors, insects, fungi, and bacteria must also be kept in mind.

Drought may affect the tree and open it to the attack of parasites without itself having an easily noticeable influence on the health of the tree. Armillaria mellea (Vahl.) Quél. is successfully parasitic only on weakened hosts (Thomas, 1934), drought being a very common weakening agent in this respect. Hibben (1962) reports higher incidence of A. mellea in stands of declining maples he studied. Fomes annosus (Fr.) Cke. is favored by drought; it rarely attacks healthy trees (Boyce 1962, pg. 108). Cytospora spp. are often associated with cankers, being natural bark inhabitants unless the host is weakened (Boyce 1962, pg. 255).

Boyce (1962, pg. 48) expressed the difficulty in assessing the influence of each of the factors interacting with drought, saying "...slow drought injury is often difficult to diagnose, the affected trees frequently succumbing to weakly parasitic fungi or insects." Experimenters have tested the influence of drought on trees in a few basic ways.

University of Wisconsin (1964) researchers chose to construct a rain-intercept platform around individual sugar maples to test the interaction of deficiency of soil moisture with recovery from defoliation. The trees had an average height of 33 feet. A circular platform 30 feet in diamenter was constructed around the trunk of each tree. The platform, with a watertight cover, sloped downward from the bole of the tree to the perimeter. Around the perimeter was a trench 20 inches wide and 3 feet deep to prevent lateral movement of soil moisture and to eliminate possible root grafts with other trees. By this method the investigators were able to decrease the average terminal growth, increase the percent of terminal branch dieback, and in general inhibit the recovery of the trees from the defoliation.

Copeland (1955) tested the effects of drought using much the same kind of rain-intercept platform.

Kriebel (1957) counted on natural drought during his experiment. He gathered sugar maples from diverse areas in its natural range and transplanted them to his nursery in Ohio. At the end of the first summer, because it was exceptionally dry, only 20% of the trees were surviving; those that survived gave significant information on the drought hardiness of sugar maples from the different geographical areas of collection.

Lord et al (1963) used a plastic ground cover around the roots of apple trees in an attempt to limit soil moisture. They found that this method conserved ground water by preventing evaporation more then it dried the soil by providing a quick run-off for rain. A plastic ground cover that was removed between rains was found to keep the ground slightly drier then the control.

Materials and Methods

To simulate extreme drought, or drought coupled with some other adverse environmental factor, a rain-intercept platform was constructed around sugar maples, Acer saccharum Marsh., in the nursery. The twenty-five trees used were planted in a square: five rows eight feet apart, each row containing five trees eight feet apart. The trees averaged fifteen feet in height and their diameters were between 1 and 1 1/2 inches at breast height. They had been purchased from Adams Nursery, Westfield, Massachusetts and transplanted to their present location two years before, in 1964. A sloping wooden frame, varying between 12 and 36 inches off the ground, was built among the trees and extended six feet beyond their perimeter. The highest part of the frame ran parallel to the center row of trees and sloped downward on each side. Clear plastic of 4 mil thickness was stretched across the frame to form a solid surface that would shed the rain, and the trees projected through this surface. The slits in the plastic through which the trees projected were kept as small as possible, usually three inches long, and were always immediately adjacent to the support framework. The water then would have a tendency to flow away from the slits toward the natural trough formed between the supports and down the slope to the edge of the shed. In this way as little water as possible was permitted to flow down the tree-trunks and under the shed. However, no waterproof bond or tape connected the tree-trunks to the plastic. For controls, trees were set aside in another part of the nursery.

Figure 1. Partially completed watershed in the nursery, showing wooden frame and plastic covering in the process of being attached.

Two sets of "Irrometers," the trade name of a kind of tensiometer made by the Irrometer Company in Riverside, California, were placed under the water shed and two more were placed among the controls to measure soil moisture. Each set consisted of three instruments: respectively 6, 12, and 24 inches in length. Each irrometer consists of a sealed, water-filled tube equipped with a vacuum gauge at one end and a porous tip on the other end. After the tip is soaked in water for a week and is throughly saturated, the instrument may be installed in the ground. The length of the tube dictates the depth to which it is installed. Thus the 12-inch instrument has a tube 12 inches long and reads soil moisture around the 12-inch depth. Once the instrument is installed in the ground, water is drawn out through the tip in proportion to the dryness of the ground and creates a vacuum in the column. This registers on the gauge, and the drier the soil the higher the reading.

The bacteria isolated in Part II were inoculated into the trees under the rain intercept platform and into the controls. The inoculation procedure was first to swab a small area of the bark with 70% ethyl alcohol. When the alcohol had evaporated a cut was made into the bark with a 1/4-inch gouge that had been cleansed in the same way. While the gouge was still in the wound, the bacteria in the nutrient broth in which they had grown were introduced into the wound from a pipette. The transpirational pull in the broken vessels pulled in some of the inoculum. The wound was left untreated.

Results

Data were collected from the tensiometers during the period from June 14 to August 25, 1966, and are presented in the charts at the end of this section. Evaluation of the inoculations will take place in succeeding years.

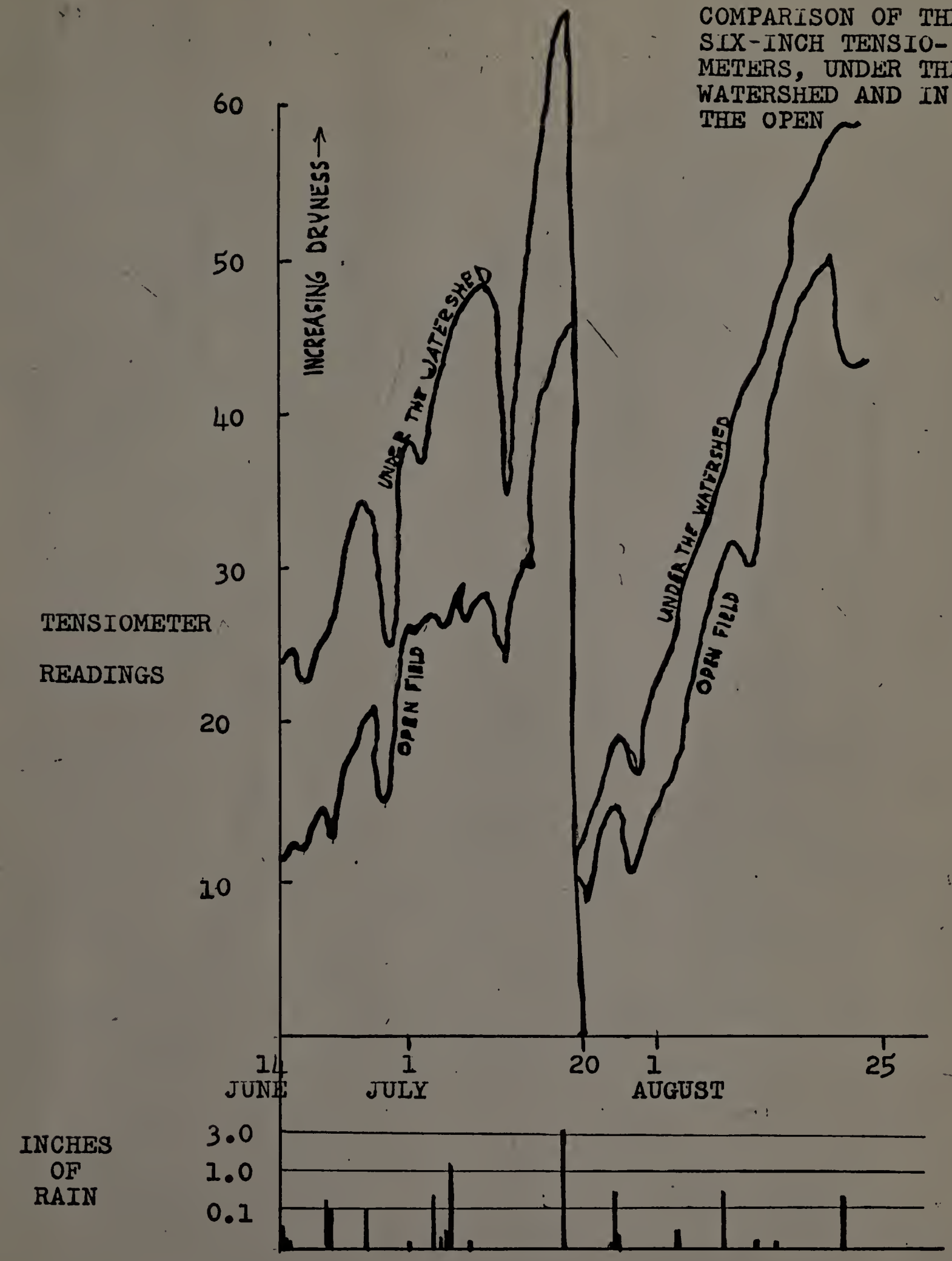
Discussion

Comparison of the readings from the tensiometers under the watershed with those in the adjoining field shows that during the summer there was less moisture in the soil under the shed. The shed was completed about June 1. Between then and when the tensiometers were installed and the readings begun, June 14, 2.75 inches of rain fell. This was 73% of the normal rain for June, or 83% of the rain that actually fell in the month. The first two weeks of June were wet. The initial difference in the readings, June 14, is small, but as the weather became drier during the last half of the month and the beginning of July the readings diverged, especially for the six-inch instruments. (Graph 3/A). Before July 20 the small rains were successfully diverted by the shed, the six-inch tensiometer in the open showing a clear response to each of these rains while that under the shed was relatively stable. The 12- and 24-inch depths showed no pronounced response to individual rains; however the cumulative effect of the shed in diverting the rain probably led to the consistently higher readings for these depths under the shed, when compared to those in the open.

This trend ended July 20 when 3.2 inches of rain fell. This rain saturated the nursery equally under the shed and in the open. At this time

GRAPH B

COMPARISON OF THE SIX-INCH TENSIOMETERS, UNDER THE WATERSHED AND IN THE OPEN



the field was awash, like a pond, and the shed was unable to keep the ground dry underneath as water flowed in around the edges uninhibited. The University of Wisconsin (1964) experimenters chose to trench the perimeter of their shed in an effort to avoid this effect, only to find that their trench retained water like a moat and fed it to the soil underneath the shed. In future uses of the shed technique, a dike around the shed's perimeter might prove the most effective way of preventing surface water from flowing under the watershed. However if the land had a bias, so that a trench could empty itself at some lowest point and not just fill up and feed the water back, it might be effective. This was not possible in the nursery at Amherst.

The data from July 21 to August 25 show the same pattern as those of the earlier period. There was a gradual increase in water tension, both in the open and under the shed, but the increase under the shed was greater. The fact that, in this period as well as the first, the water tension had a tendency to maintain a certain constant difference between the two areas suggests that the ground water moved to the soil under the shed if the difference between the two exceeded some certain amount.

Ultimately, of course, the success of the shed technique for inducing drought would be judged by the symptoms of drought on the trees underneath. The obvious foliar symptoms of drought did not appear. However, success of the inoculations, which may be dependent on the slight increase in water tension that did occur, awaits assessment at a later date.

TENSIOMETER READINGS FOR THE MONTH OF JUNE

OPEN FIELD

DEPTH \ DAY	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
6"	12	12	10	14	14	14	12	15	16	17	18	19	13	14	19	22	24
12"	11	11	10	13	13	13	12	14	14	15	16	16	14	14	14	16	18
24"	12	12	12	12	13	13	13	14	14	15	16	16	14	14	16	16	18
6"	10	12	12	13	14	16	12	18	21	22	22	24	15	17	26	28	30
12"	11	12	10	13	14	14	13	14	16	16	17	17	15	15	18	19	20
24"	12	11	10	13	13	13	13	14	14	14	16	16	14	14	16	16	18
						UNDER THE WATERSHED											
6"	17	20	18	20	21	22	22	27	29	30	30	31	22	23	30	34	36
12"	14	15	13	18	18	16	18	18	20	20	20	21	18	18	22	22	24
24"	14	14	14	15	15	16	17	18	18	18	19	19	19	19	20	21	22
6"	25	30	25	28	29	31	33	36	38	39	36	37	26	28	38	42	40
12"	14	14	13	16	16	16	18	18	18	20	21	21	17	18	21	22	24
24"	12	14	13	15	15	16	17	17	20	18	20	21	17	18	20	21	22
						PRECIPITATION											
	.06	.03	.02			.21	.11					.12					
	am pm	pm	am pm			pm	am					pm					

TENSIOMETER READINGS FOR THE MONTH OF JULY

OPEN FIELD

DEPTH	DAY 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
6"	23	23	24	22	24	26	20	20	22	20	16	18	24	22	28	35
12"	17	17	18	17	18	18	18	17	18	18	14	14	17	20	29	37
24"	16	16	16	16	16	16	16	16	16	16	16	15	16	18	19	19
6"	28	29	30	27	29	33	30	33	34	32	25	27	36	42	44	40
12"	19	19	20	21	21	22	18	23	24	36	25	27	31	38	38	45
24"	16	18	20	17	19	19	18	20	21	21	21	21	24	25	25	27
				UNDER THE WATERSHED												
6"	36	38	37	39	42	44	48	53	50	45	35	37	40	49	52	61
12"	24	25	26	26	27	28	30	30	31	33	29	32	38	40	46	50
24"	22	23	25	27	27	28	29	29	20	21	21	20	19	20	20	21
6"	37	44	46	49	50	52	48	45	46	48	32	33	38	45	59	57
12"	23	23	24	23	25	25	30	30	30	30	28	35	42	43	48	50
24"	22	22	23	23	24	24	28	27	27	27	26	26	26	26	26	27
						PRECIPITATION										
				.01			.31		.02	.04	.65		.01			
				am			am		pm	am	am		am			

TENSIOMETER READINGS FOR THE MONTH OF JULY

OPEN FIELD

DEPTH \ DAY	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
6"	42	43	45	0	7	2	4	5	7	10	12	8	9	12	13
12"	36	37	40	0	4	10	11	12	14	13	14	12	14	14	13
24"	22	23	24	0	7	12	12	13	13	14	14	14	14	14	15
6"	40	45	51	0	10	12	14	15	17	18	19	12	10	14	15
12"	44	45	46	2	10	13	16	16	17	19	17	15	15	17	19
24"	29	28	30	4	10	12	12	13	13	15	16	15	15	14	16
						UNDER THE WATERSHED									
6"	63	63	65	0	10	14	16	17	18	17	19	17	15	19	24
12"	57	56	60	4	10	12	11	12	14	13	12	10	10	11	12
24"	21	22	22	12	10	12	12	13	13	12	13	13	12	14	14
6"	65	72	78	0	6	10	12	14	16	19	20	16	16	18	20
12"	57	61	64	0	3	6	6	12	13	17	21	21	20	22	23
24"	28	30	31	0	2	5	7	8	10	14	16	15	16	15	14
						PRECIPITATION									
				.320	.01						.03	.48	.07		
				am	am						am	am	am		

TENSIOMETER READINGS FOR THE MONTH OF AUGUST

OPEN FIELD

DEPTH	DAY 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
6"	13	15	14	17	18	20	19	24	28	30	26	28	29	31	36	35	39	44	50	47	52	51	50	45	44
12"	14	15	16	16	17	18	19	20	22	21	22	24	24	30	31	32	33	34	35	39	40	42	43	40	41
24"	15	15	15	15	16	16	16	17	17	17	18	17	18	18	19	19	20	20	21	20	21	21	21	21	21
6"	15	17	19	20	24	28	31	29	32	34	35	31	31	34	35	36	39	43	45	46	47	48	48	40	41
12"	18	19	19	20	20	21	21	23	24	24	25	24	23	24	27	31	34	36	38	40	38	39	39	38	38
24"	16	16	16	15	14	15	15	16	17	17	18	18	19	19	20	20	21	21	23	22	22	23	23	24	23

UNDER THE WATERSHED

DEPTH	DAY 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
6"	25	26	29	30	34	31	32	39	37	41	44	48	49	51	54	53	55	59	58	61	60	62	63	62	61
12"	12	12	13	14	15	17	19	21	22	22	23	25	27	29	31	34	37	41	42	43	44	46	48	48	49
24"	14	14	15	15	13	13	14	15	15	16	17	17	18	18	19	19	20	21	23	23	23	24	24	24	29
6"	20	21	23	27	31	32	34	29	33	35	37	36	36	39	40	44	46	47	49	51	53	54	55	55	56
12"	22	22	24	26	26	27	28	29	31	32	32	33	32	30	34	36	38	43	45	46	47	49	50	49	50
24"	15	15	16	14	16	17	18	17	18	19	20	20	21	24	21	24	25	24	23	25	26	28	28	27	27

PRECIPITATION

	.05									.21			.02	.04					.0328						
	am									am			am pm	am pm							am pm		pm		

Approved by:

Robert P. Matlick

Francis W. Holmes

Thesis Committee

Head of Department

Date May 15, 1967